Reprinted from JOURNAL OF BACTERIOLOGY
Vol. 76, No. 1, pp. 1-14, July, 1958
Printed in U.S.A.

REGULATION OF ENZYMATIC ACTIVITY IN THE INTACT CELL: THE β-D-GALACTOSIDASE OF *ESCHERICHIA COLI*[1]

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Received for publication November 1, 1957

Large differences in enzymatic activity are often found between intact cells and extracts. Lacking a better explanation, it has frequently been assumed that the penetration of the substrate is the rate limiting step in the intact cells. The experiments reported here were designed to test several hypotheses which might account for the discrepancy.

The β-galactosidase (lactase) in *Escherichia coli* was selected as a model system because of the sensitivity and ease of assay, together with the extensive knowledge of the induction, isolation, and general properties of the system (Lederberg, 1950; Cohn and Monod, 1951; Kuby and Lardy, 1953; Rotman and Spiegelman, 1954). Deere (1939) reported that drying *E. coli-mutabile* appeared to "activate" the lactase. Lederberg (1950) studied the β-galactosidase of *E. coli* strain K-12 with a chromogenic substrate and found discrepancies of 10- to 47-fold between the activity of the intact cells and of disrupted cells or extracts. In addition, Lederberg found that the activity of the intact cell was protected from pH changes and inhibitory cations.

The hypotheses tested in this investigation were: passive diffusion, inhibitors complexed with the enzyme, and product inhibition of the enzyme. All three were ruled out by the data presented below. Consequently, a more compatible proposal is given which assumes the existence of a specifically induced penetration mechanism. During the course of this work, Monod and his associates postulated an inducible enzymelike mechanism for specific control over galactoside penetration and termed the agent a "permease" (see Cohen and Monod, 1957). The experiments described here were completed before the data supporting the permease hypothesis became available and therefore were not designed to test this hypothesis. Nevertheless, when our findings and the permease data were compared, basic similarities and differences between the two hypothetical penetration systems became apparent. Possible ways of reconciling the two views are discussed.

[1] Paper no. 676 of the Department of Genetics. Supported by an American Cancer Society Institutional Grant and by a grant from the United States Public Health Service.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. To avoid the additional variable of adaptation, the strain W-1317, a constitutive mutant (K-12) of *E. coli* was employed for most of the experiments. This mutant, obtained by selection on neolactose agar, synthesizes large amounts of β-galactosidase in the absence of external inducer (Lederberg, 1951). Experiments involving adaptation were performed with the parent K-12 stock. Davis' (1949) minimal medium with 0.1 per cent glycerol as the carbon source was used throughout. Unless otherwise specified, the cells were grown in continuous culture in a modified chemostat (Rotman, 1955*a*) with an average generation time of 3 hr. For most of the experiments, 12 ml of culture were withdrawn from the growth tube. The cells were then washed twice with 20 ml of cold 0.02 M sodium phosphate buffer at pH 7.2 by centrifugation at 14,000 × G for 3 min in the high speed head of an International centrifuge. The cells were resuspended in 12 ml of the same buffer to give an average nitrogen content of 70 μg per ml.

Some experiments required the use of bacteria freed of exogenous salts. Repeated washing with water proved inefficient in that the bacteria ceased to pack in the centrifuge even at high speed. Consequently, the cells were passed through an ion exchange column as described by Rotman (1956). The deionization procedure and subsequent storage in distilled water at 0 C did not impair cell viability and recoveries were

quantitative. After this treatment, less than 0.04 ppm of sodium or potassium were found to be present in the supernatant by flame photometry.

Chemical reagents.[3] *o*-Nitrophenyl-β-D-galactopyranoside was synthesized according to the method of Seidman and Link (1950) or procured from Mann Laboratories. *p*-Nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-L-arabinopyranoside, and *o*-nitrophenyl-α-L-arabinopyranoside were kindly supplied by Dr. K. P. Link. Methyl-β-D-thiogalactopyranoside was obtained through the kind assistance of Dr. A. Novick. Galactose (Pfanstiehl) was recrystallized four times from aqueous ethanol and dried at 60 C under vacuum for 24 hr. Lysozyme and ribonuclease were obtained from Armour Co. The lactose was from Difco Laboratories, but all other reagents were analytical grade. Water was deionized and then glass distilled.

Analytical methods. β-D-Galactosidase was measured with ONPG as a chromogenic substrate (Lederberg, 1950). The test solution (0.05 to 0.2 ml) was added to 2 ml of M/1000 ONPG dissolved in the buffer described above. The reaction was stopped after 1 to 15 min incubation at 30 C by addition of 10 ml of 0.1 M sodium carbonate. The *o*-nitrophenol was determined colorimetrically at 420 mμ. Enzymatic activity was expressed as mμmoles ONPG hydrolyzed per minute.

Benzene-treated cells were prepared by shaking 2 ml of a washed suspension of cells with 0.1 ml of benzene. After incubation for 10 min at 30 C, 0.05 ml was withdrawn from the tube and assayed as indicated above. A 0.2 ml sample of the untreated suspension was used to assay the enzymatic activity of the intact cells. The cells were centrifuged and removed after stopping the reaction with sodium carbonate. Blanks were prepared by adding the cells after the sodium carbonate. The enzymatic activity obtained when cells were treated with benzene was taken as the standard. The activity of the intact cells showed linear kinetics on a Michaelis-Menten plot up to a concentration of 3.4×10^{-3} M ONPG, thereby confirming and extending Lederberg's (1950) original observations.

Ribose was determined by the orcinol test (Kerr and Seraidarian, 1945) and desoxyribose by the diphenylamine test (Sevag *et al.*, 1940). Separation of nucleosides, nucleotides, and free bases was accomplished by the method of Cohn (1950). Protein was measured by the biuret reaction (Gornall *et al.*, 1949).

[3] Abbreviations: ONPG = *o*-nitrophenyl-β-D-galactopyranoside, DNA = desoxyribose nucleic acid, RNA = ribose nucleic acid, β-galactosidase = β-D-galactosidase.

EXPERIMENTAL RESULTS AND CONCLUSIONS

Increase in enzymatic activity by treatment with solvents. (1) Benzene—In agreement with other investigators (Lederberg, 1950; Koppel *et al.*, 1953) treatment of the cells with benzene or toluene in 0.02 M Na-phosphate buffer, pH 7 to 7.3, yielded enzymatic activity similar to that obtained with cell-free extracts prepared by sonic disintegration or drying *in vacuo*. Five minute treatments with 5 per cent (v/v) benzene, including shaking once or twice by hand, brought the enzymatic activity of the suspension to its maximal value without releasing more than 5 per cent of the enzyme into the supernatant. The bulk of the enzyme was retained by the "cells." Vigorous mechanical shaking provided maximal activity in about 15 sec at room temperature. The reaction appeared to be temperature independent in the range 0 to 40 C.

Benzene-treated cells do not appear different from normal cells under phase contrast microscopy, but when Giemsa stain is used the treated cells are less basophilic than the normal ones, suggesting the loss of ribonucleic acid.

Under the electron microscope the difference becomes evident as shown in figure 1. The destruction of the cell wall in the benzene-treated cells is quite apparent but the possibility that the drying process might have increased the damage must be considered.

In contrast with the β-galactosidase of intact cells, the benzene-treated cells and the free enzyme were similar in respect to pH optimum, K_s value, and inhibition by alkali metal ions. The only detectable difference was that in our hands benzene-treated cells did not bind significant amounts of specific rabbit antienzyme. Throughout this work, therefore, benzene-treated cells were used as reference for assay of total enzymatic activity.

(2) Other solvents—The low solubility in water of benzene or toluene limits somewhat the usefulness of these solvents. Therefore a search for a more suitable agent was conducted. Isoamyl alcohol was chosen because of its higher solubility at low temperatures and because it has a low

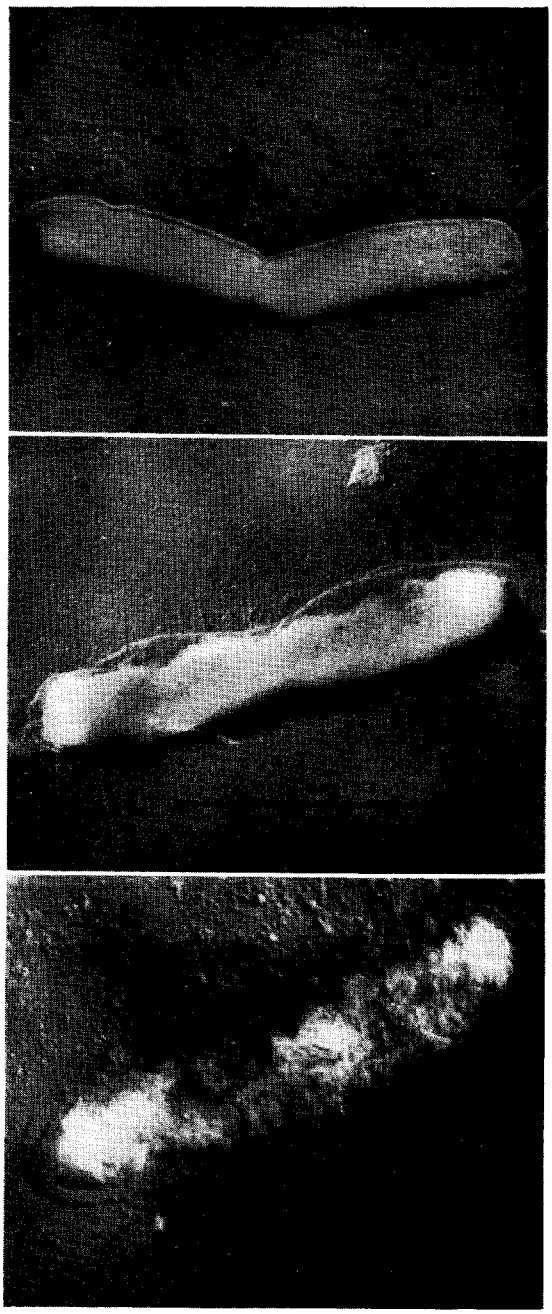

*Figure 1.** *Top.* Normal cells of *Escherichia coli*. *Center.* Cells after 10 min treatment with benzene. *Bottom.* Benzene-treated cells as above but incubated 10 more min with substrate, alizarin-β-D-galactopyranoside.

* Uranium shadowing. Electron photomicrographs courtesy of Dr. Paul J. Kaesberg.

ultraviolet absorption and would not interfere with the spectrophotometric estimation of purines and pyrimidines. Table 1 shows that only relatively insoluble hydrocarbons or aliphatic alcohols give maximal activation under the conditions employed.

Since isoamyl alcohol disappears during the process of activation, a minimum of about 6×10^{-12} g of isoamyl alcohol per cell was necessary at 0 C for maximal enzymatic activity.

Physiological state of the cells influencing enzymatic activity. From Lederberg's (1950) work and our earlier experiments it was evident that the ratios between enzymatic activities of extracts and intact cells were not reproducible. As shown in table 2, the variable was found to be the age of the cultures. Stationary cells show nearly the same enzymatic activity as their extracts, whereas cells in the exponential phase of growth exhibit only a small fraction of their potential activity, e. g., the activity ratio between the extract and the intact cells is 50 to 100. By using a chemostat and keeping the culture in constant growth it was possible to obtain reproducible values for the activity ratio. Furthermore, it was established that large variations in growth rate in the chemostat did not influence this ratio significantly (table 2). This observation supports the assumption that in the steady state the vast majority of the cells are actively growing even at low generation times.

TABLE 1

Increase in enzymatic activity of cultures by treatment with solvents

One ml of bacterial suspension, prepared in phosphate buffer as described in Methods, was incubated for 10 min with 0.2 ml of the solvent tested. The β-galactosidase activity is expressed as per cent of the activity obtained with benzene.

Solvent	β-Galactosidase
	%
No addition	3.6
Benzene, toluene, xylene	100
Ethyl alcohol	33
s-Butyl alcohol	—
t-Butyl alcohol	52
1-Pentanol	97
Isoamyl alcohol	96
Octyl alcohol	58
5% Phenol	32
5% Na Lauryl sulfate	37
Ethyl ether	78
Acetone	8

TABLE 2

Relationship between age of cultures and enzymatic activity of whole cells

β-Galactosidase activity was determined in washed cells with and without benzene treatment as described in Methods. The activity is expressed as the ratio between benzene-treated cell and intact cell activities.

Growth Conditions and Age of Cultures	Ratio β-Galactosidase Activity
Stationary, aerated, 12 hr	21
Stationary, not aerated, 24 hr	24
Stationary, not aerated, 37 hr	5
Stationary, not aerated, 47 hr	3
Chemostat, 1 hr generation time	173
Chemostat, 1.4 hr generation time	166
Chemostat, 7.7 hr generation time	124
Chemostat, 11.1 hr generation time	161

Passive-diffusion hypothesis. Induced biosynthesis of enzyme (adaptation) and cellular expression of enzymatic content. An opportunity to test a simple passive diffusion hypothesis is offered by the fact that the ratio of intact cell activity to extract activity changes with the β-galactosidase content of the cell (Rickenberg *et al.*, 1953). The β-galactosidase content per cell can be altered (Monod *et al.*, 1951) by varying the concentration of a suitable inducer of the enzyme in the wild type *E. coli*.

The proposed diffusion hypothesis postulates that the rate of penetration of the substrate is the rate-limiting step when the cell expresses only a fraction of its enzyme content. The hypothesis was tested under the following assumptions: (a) Inasmuch as passive diffusion is under test, it can be assumed that the rate of penetration is constant if conditions of "gratuity" are used throughout the experiment, i. e., the medium has a constant chemical composition except for the concentration of the nonmetabolized inducer (Monod and Cohn, 1952). Other possible penetration mechanisms which fulfill this assumption will not be discriminated by our test; (b) The physical properties of the enzyme inside the cell are similar to those of the enzyme in cell-free extracts; and (c) The concentration of substrate around the cell remains practically constant,

i. e., diffusion rate in the medium is much larger than the entry rate of the substrate. This assumption is valid because benzene-treated cells exhibit activities up to 170-fold larger than intact cells. If we recall that a benzene-treated cell contains the same amount of enzyme enclosed in practically the same volume as an intact cell, we have to conclude that the rate at which substrate reaches the periphery of the cell is not the limiting step. It should be noted that for the purpose of our discussion "penetration of the cell by the substrate" could mean also arrival of the substrate at the enzyme site.

The same concentration of substrate was used in all the assays and it remained practically constant throughout the experiment.

The hypothesis thus formulated predicts the curves illustrated in figure 2*A*. The predictions are: (a) At low levels of induction, the intact cell should express its full enzyme content until substrate penetration becomes rate limiting, i. e., throughout this range, enzymatic activity of cells and extracts should be the same, and, (b) After substrate penetration becomes rate limiting as indicated by the lower activity of cells compared with extracts, the enzymatic activity of the intact cells should remain constant. Therefore the ratio of extract activity to cell activity should increase progressively with the increase in the enzyme content of the cell.

A test of these predictions is shown in figure 2. The experimental curves in figure 2*B* differed radically from the theoretical (2*A*). In no portion of the former was either prediction satisfied. Thus the intact cell expressed little or none of the enzyme content when this was small and at higher induction levels expressed a constant fraction of the enzyme synthesized. The intact cell activity did not reach a plateau but increased steadily with increasing enzyme content. Finally, the ratio of extract to cell activity reached a maximum long before the enzyme content did. Thus one must conclude that the passive diffusion hypothesis as stated here is incompatible with the facts. Any other hypotheses that do not involve a change in the rate of substrate entry during enzyme induction would be equally incompatible.

The same test was applied to the constitutive strain which synthesizes as much enzyme in the absence of inducer as does the parental stock in the presence of optimal concentrations of potent inducers. The enzyme content of the constitutive strain was varied by inhibition of the β-galactosidase synthesis with lactose. It was found that the lower the galactosidase content, the larger was the fraction of enzymatic activity expressed by the intact cells. Therefore passive diffusion here too must be ruled out as a possible penetration mechanism.

The enzyme complex hypothesis. The second hypothesis to be tested was the existence in the cell of an enzyme complex which reduced enzymatic activity. In this case, the increased activity of extracts or benzene treated cells would result

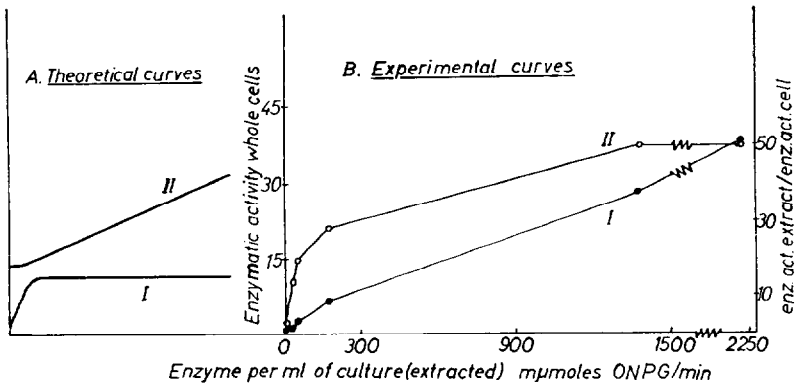

Figure 2. β-Galactosidase activity of whole cells as a function of their β-galactosidase content. Theoretical curves constructed according to the passive-diffusion hypothesis described in the text. *Curve I.* β-galactosidase activity of whole cells in mμmoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per min per ml of culture. *Curve II.* Ratio β-galactosidase activity of extract over β-galactosidase activity of whole cells. Increasing concentrations of lactose or methyl-β-D-thiogalactopyranoside were used to induce the cultures.

from the dissociation of the complex. Dissociation could occur when the internal milieu of the cell was no longer protected from external exchange.

Previous experiments (Rotman, 1955*b*) had suggested that RNA might be the complexing agent. In the present work, the test of this supposition was confined to (a) attempts to isolate the hypothetical enzyme-RNA complex from the cells and to show its *in vitro* dissociation with a comparable increase in enzymatic activity, and (b) attempts to demonstrate the inhibitory power of the RNA *in vivo* and *in vitro*.

The hypothesis could be extended to include other complexing molecules such as the enzyme itself (Kaplan, 1954; Bonner, 1955). A strict test of the latter however would require methods to analyze intracellular structure not available at present.

Leakage of breakdown products of RNA. As mentioned before, the lowered basophilia of benzene-treated cells suggested a substantial loss of nucleic acids. Spectrophotometric and chemical analysis confirmed that as much as 80 per cent of the total RNA content of the cell was present outside. The RNA loss was accounted for by comparable amounts of breakdown products of RNA. Small amounts of free bases and polymerized material were among the breakdown products. The relative composition of products depended upon the conditions of the benzene treatment. No detectable amounts of DNA or protein were found outside the benzene cells. Isoamyl alcohol, like benzene, caused a release of breakdown products of RNA.

RNA breakdown products were also found to be released from the cells upon aging with a concomitant increase in enzymatic activity. The cells remain viable throughout the aging process. Borek *et al.* (1955) reported also that the viable count of a cell suspension can remain constant while nearly 40 per cent of the RNA is excreted as breakdown products in the supernatant.

During aging, addition of Mg^{++} or Ca^{++} ions in 8.2×10^{-4} M concentration prevented the leakage of breakdown products of RNA as well as the increase in enzymatic activity. Storage in distilled water at 0 to 4 C was also effective in preventing leakage.

The relationship between RNA release and increase in enzymatic activity of the intact cells was tested further using lysozyme, polymyxin B, and heating at 51 C. Polymyxin (Newton, 1956) and heating at 51 C (Califano, 1952) have been previously shown to cause leakage of depolymerized form of RNA. Lysozyme alone has not been reported to cause any direct effect on *E. coli* at neutral pH, although recently it has been shown to cause lysis at pH 7.6 in the presence of Versene (Repaske, 1956). Treatment with lysozyme released half of the RNA in polymerized form precipitable by $HClO_4$. This effect of lysozyme was inhibited 70 per cent by 8×10^{-5} M uranyl acetate.

Furthermore, when the release of RNA by polymyxin was inhibited by high concentrations of the antibiotic or by divalent cations (Newton, 1956) the enzymatic activity was inhibited proportionally. Cells treated with either lysozyme or polymyxin exhibited about 15 per cent of the maximal β-galactosidase activity. On the other hand, when both agents were used in the order indicated maximal activity was obtained. Reversing the order or adding the two together resulted in reduced activity (table 3). Cells heated at 51 C did not show additive effects with either lysozyme or polymyxin. Lysozyme treatment resulted in no detectable loss in viable cells, whereas 14 min at 51 C killed 85 per cent of the cells and polymyxin left only a few survivors.

As shown in figure 3, treatment with any of these agents resulted in a release of RNA proportional to the increase in β-galactosidase.

Although this proportionality between RNA excretion and β-galactosidase activity was found within every set of experiments involving a single activating agent, the amount of RNA released did not correspond to the same enzymatic activity for experiments with different activators. For instance, when isoamyl alcohol released 237 μg of RNA/mg dry cells with an increase in enzymatic activity of 4520 mμmoles ONPG/min mg dry cells, aging in 0.02 M sodium phosphate buffer for 8 hr released 23.6 μg of RNA/mg dry cells with an increase of 119 mμmoles ONPG/min mg dry cells.

This lack of correlation between activating agents suggests that the release of RNA and the increase in enzymatic activity could be the result of a more general effect, i. e., loss of permeability, rather than a combination of RNA and enzyme as postulated before (Rotman, 1955*b*). Further evidence of this hypothesis is given below.

Preparation of inhibited enzyme in vitro. It was

TABLE 3

Synergistic effect of lysozyme and polymyxin upon the increase in enzymatic activity of whole cells

Chemostat-grown cells were washed and resuspended in phosphate buffer as described in Methods. Three tubes containing 2 ml of cell suspension plus the additions indicated below were incubated 30 min at 37 C. After this first incubation, 0.1 ml of each tube was used to determine β-galactosidase activity and the rest of the cells were spun down and resuspended in 2 ml of the same buffer. Aliquots of each tube, with and without additions, were incubated at 37 C for another 30 min. β-Galactosidase was determined in each aliquot at the end of this second period of incubation. The results are expressed as per cent of the β-galactosidase activity of benzene-treated cells. Activity of cells before incubation at 37 C was 1.1 per cent. Activity of supernatants from tubes 2 and 3 after the second incubation was 1.2 and 0.3 per cent, respectively.

Tube No.	Additions	β-Galactosidase Relative Rate
First incubation		
1	0.2 ml H_2O	4.7
2	0.2 ml polymyxin B (0.5 mg/ml)	22.6
3	0.2 ml lysozyme (12.5 mg/ml)	14.9
Second incubation		
1	No additions	8.0
2	No additions	22.6
2	0.1 ml lysozyme (50 mg/ml)	57.5
3	No additions	20.0
3	0.1 ml polymyxin B (0.5 mg/ml)	100.5

possible to prepare extracts containing β-galactosidase in an inhibited form. The inhibition could be overcome by addition of salts or by incubation at 37 C (figure 4). The key to the preparation of inhibited extracts is to avoid the use of salts throughout the extraction procedure. The cells were collected in a refrigerated Sharples centrifuge, and after three washings with water at 15,000 rpm in a Spinco (head no. 20), they were ground with dry ice and lyophilized. The extracts were prepared simply by grinding 0.5 g of dry powder in a cold mortar with 5 ml of cold water

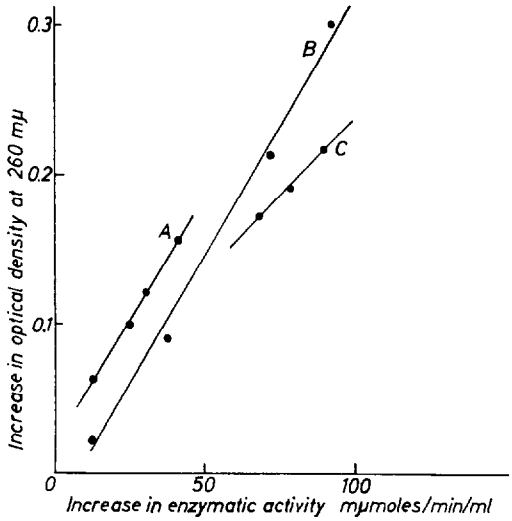

Figure 3. Relationship between release of RNA-breakdown products in the supernatants and activation of the β-galactosidase of whole cells. *A*. Cells aged in 0.02 M $Na\text{-}PO_4$ (pH 7.35 at 26 C) for 3 hr at 37 C. *B*. Cells treated with polymyxin B in decreasing concentration. The lowest increase in activity corresponds to 830 μg of polymyxin per ml, the highest to 210 μg per ml. *C*. Cells heated at 51 C in 0.02 M $Na\text{-}PO_4$ (pH 7.2 at 25 C) for 15 min.

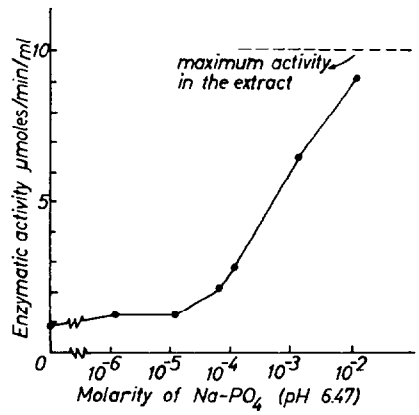

Figure 4. Increase in the β-galactosidase activity of inhibited cell-free extracts by addition of salts. See the text for preparation and assay of inhibited extracts. The pH of the buffer was measured at 0 C and it was chosen to match the pH of the extract.

and eliminating the debris by 8 min centrifugation at 10,000 rpm in the high speed head of an International centrifuge.

These extracts were assayed at 0 C by adding

0.05 ml of 0.05 M ONPG (previously equilibrated at 0 C) to 0.1 ml of extract and stopping the reaction with 10 ml of 0.1 M Na_2CO_3. The assay was proportional to incubation time up to 6 min.

β-Galactosidase activity in these inhibited extracts was not diminished by 60 min centrifugation at 40,000 rpm in the no. 20 head of a Spinco centrifuge. Thus, the association of the enzyme with a particulate matter is ruled out as a cause of inhibition.

That the inhibition in these extracts is not of the known type produced by alkali metal ions (Lederberg, 1950) was shown by the fact that the initial salt concentration affected the enzymatic activity of aliquots of extract reaching identical final conditions prior to assay (figure 4).

The relationship between the inhibited extracts and the physiological problem in question is obscure because the cells which had been treated previously with isoamyl alcohol, and were therefore capable of expressing their entire enzyme content, nevertheless yielded inhibited extracts.

Inhibitors of β-galactosidase released by the cells. According to the enzyme-complex hypothesis the complexing agent should be found free after activation of the enzyme. An unsuccessful search for the complexing agent was conducted among the excretion products of cultures which had leaked most of their RNA with a proportional increase in β-galactosidase activity. A 5-L culture of the constitutive mutant was treated with isoamyl alcohol and the supernatant was concentrated from the frozen state under vacuum.

Several fractions obtained by precipitation of the concentrated supernatant with ethanol were tested for inhibition of cell-free enzyme or benzene-treated cells. None was found inhibitory. Likewise yeast RNA was not inhibitory in either system. The effect of highly polymerized RNA from *E. coli* could not be determined. No reports of a successful method of preparation could be found in the literature. The difficulty probably stems from the presence of active RNAase(s) in the extracts (Manson, 1953).

The product inhibition hypothesis. This hypothesis explains the reduced activity of the intact cell in terms of inhibition of the enzyme by the reaction products. Galactose, one of the products, is known to be a competitive inhibitor of the enzymatic hydrolysis of ONPG. High concentrations of galactose could accumulate in the intact cell if its excretion rate happened to be smaller than the hydrolysis rate of ONPG. The increasing concentration of galactose would inhibit the enzymatic reaction progressively until a steady state had been reached in which the rates of hydrolysis and excretion of galactose remained constant. This hypothesis predicts that the initial enzymatic activity of the intact cell before accumulation of reaction products would be larger. The prediction was tested by following the initial rates of hydrolysis of ONPG by a concentrated cell suspension in a recording spectrophotometer of very high sensitivity. A double beam apparatus which recorded the difference in optical density between 440 and 480 mμ was used (Chance, 1956). These experiments were performed in collaboration with Dr. Britton Chance at the University of Pennsylvania.

The data summarized in figure 5 show that a steady rate of hydrolysis had been attained by the

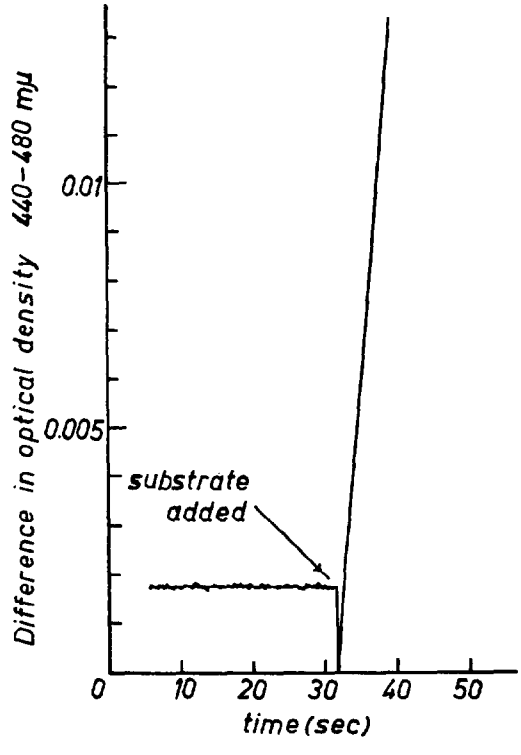

Figure 5. Hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) by intact cells measured in a double-beam recording spectrophotometer. The instrument recorded the difference between optical densities at 440 and 480 mμ.

cells as early as one second after substrate addition. Extrapolation to zero time permitted the estimate that the concentration of *o*-nitrophenol (and therefore of galactose) in the total suspension at the end of the first second could not have exceeded 1×10^{-6} M. Since the product inhibition hypothesis would demand that a steady state of enzymatic inhibition should already have been established at this time one may ask if the amount of galactose that was present was sufficient to have brought this about. The intracellular volume of the suspended cells was determined to be 6.3×10^{-3} ml per ml suspension. Thus even were all the galactose that had been produced in one second still within the cells, its concentration could have been no more than $(1 \times 10^{-6})/(6.3 \times 10^{-3}) = 1.6 \times 10^{-4}$ M. But the data of Kuby and Lardy (1953) for the *in vitro* inhibition of highly purified β-galactosidase showed that 0.4 M galactose is required for 95 per cent inhibition in the presence of 2.28×10^{-4} M ONPG (the amount used in the present experiments). It follows then that this concentration of galactose is $(0.4)/(1.6 \times 10^{-4}) = 2500$-fold greater than could have been generated in the cells in one second. In other words, the amount of galactose that actually was present would have had to be crammed into $1/2500$ of the total cell volume to have been inhibitory. (Assuming a significant effusion of galactose from the cells during this time, the fraction would be even lower.) But this is patently impossible when it is considered that the highly purified β-galactosidase represents 0.5 to 1 per cent of the total cell protein (Rotman and Spiegelman, 1954) so that the volume of enzyme itself is greater than $1/2500$ of the total cell volume.

It should be noted that the only assumptions underlying this argument are that the enzyme inside the cell behaves as the purified enzyme and that the substrate concentration inside the cell is the same as that outside.

Penetration mechanism hypothesis. *p*- and *o*-Nitrophenyl-α-L-arabinopyranoside have also been found to act as substrates for the β-galactosidase (Lederberg, *private communication*). These compounds have the same ring structure as the β-D-galactopyranosides. They differ strikingly in that intact cells exhibited the same activity against them as did extracts (table 4). The full expression of enzyme content by the intact cell excludes immediately the idea of an altered (Kaplan, 1954), inhibited (Rotman, 1955*b*), or stacked enzyme (Bonner, 1955). It is not likely that the enzyme would be compounded in such a form that binding sites for the arabinosides would be free but those for the galactosides would be obstructed. The same reasoning applies to the stacked enzyme.

In addition, the difference in behavior of the intact cell toward the two classes of substrates cannot readily be ascribed to different rates of passive diffusion because they are so similar structurally. It therefore seems more likely that a se-

TABLE 4

Effect of Na and K ions in the hydrolysis of β-D-galactosides and α-L-arabinosides by β-galactosidase

β-Galactosidase activity was determined as described in Methods at 10^{-3} M substrate concentration. Na or K phosphate (0.02 M) buffer pH 7.2 (25 C) were used in the assay mixture. The results on the effect of ions are given as the ratio β-galactosidase activity in Na^+ buffer over β-galactosidase activity in K^+ buffer. The values for induced wild type whole cells are the results of a single experiment.

Culture	Ratio Na^+ Activity/K^+ Activity		mμmoles of Substrate Hydrolyzed per min per μg N in Na^+ Buffer	
	Substrate		Substrate	
	ONPG*	ONPA*	ONPG*	ONPA*
Constitutive whole cells	1.03	3.30	1.73	0.72
Wild type induced whole cells	1.01	3.35	1.79	0.62
C_6H_6-treated constitutive	1.75	5.55	66.7	1.69
C_6H_6-treated induced wild type	1.83	4.10	57.8	1.36
Noninduced wild type whole cells	1.35	—	0.03	Undetectable
Purified β-galactosidase	1.78	5.60	960	27.9

* ONPG = *o*-nitrophenyl-β-D-galactoside; ONPA = *o*-nitrophenyl-α-L-arabinoside.

lective penetration mechanism is involved. To account for the increase in enzymatic activity of the intact cell with increase in enzyme content, one would have to assume that the penetration mechnism is induced together with the enzyme (figure 2*B*). Because of its inducibility and specificity, one might visualize the penetration mechanism as enzymatic in nature although it could equally well act as a specific "keyhole" at the cell surface.

Further evidence in support of the above hypothesis was obtained by a study of metal ions known to inhibit the galactosidase *in vitro*. Lederberg (1950), using ONPG as a substrate, reported that Na ions did not affect the intact cell activity although they could activate β-galactosidase in extracts as compared with K ions. We have extended these results using *p*-nitrophenyl-β-D-galactopyranoside. Again, no effect of ions upon intact cell activity toward this substrate could be noted but the effects of Na and K were precisely reversed in extracts. When the arabinosides were employed, metal ions were found to influence both intact cells and extracts (table 4). A similar situation was encountered in noninduced cells with only background enzyme.

Regardless of the nature of the substrate, intact cells did not exhibit any β-galactosidase activity whatever when assayed in distilled water. The activity was restored upon addition of maleic or phosphoric buffers. Although Rb, K, Na, Li, etc. were innocuous toward intact cells when they were acting upon β-galactosides, triethanolammonium ions were potent inhibitors. Kuby and Lardy (1953) reported that this ion inhibits the purified β-galactosidase.

From these results it seems that the metal ions are inert to the intact cell activity on β-galactosides not because the ions cannot reach the enzyme site, but because they do not interfere with the penetration mechanism. This idea is reinforced by the inhibitory effect of Tris buffer which should not exist if the ions cannot reach the enzyme site. The α-L-arabinosides would have such affinity for the β-galactoside penetration mechanism as to permit a relatively rapid entrance. Thus, for the arabinosides, the β-galactosidase would be the rate limiting step *in vivo*. The possibility of a separate penetration mechanism specific for the arabinosides can not be excluded. Nevertheless, its postulation adds nothing to our hypothesis. On the contrary, to set aside the β-galactoside penetration mechanism, it would be necessary to accept the existence of a specific penetration mechanism for arabinosides but not for galactosides.

The fact that intact cells placed in a mixture of ONPG and 0.1 M Na_2CO_3 still can hydrolyze a minute amount of ONPG is additional evidence in favor of the penetration mechanism. The experiment indicates that ONPG penetrates the cell faster than OH^- ions do. Similarly, HCl, trichloracetic acid, or formaldehyde do not stop the hydrolysis *in vivo* completely.

The inference from the α-arabinoside experiments is valid only if the β-galactosidase is the main enzyme capable of splitting α-L-arabinosides. This assumption is strongly supported by two lines of experimental data: (a) The ratio between the rates of hydrolysis of *o*-nitrophenyl-α-L-arabinopyranoside and ONPG, under constant conditions, is the same for benzene-activated cells, crude extracts, or purified β-galactosidase. The average ratio at 10^{-3} M concentration of substrate for benzene-activated cells was 39.4 and for purified enzyme, 34.3. The ratio given by Kuby and Lardy (1953) for highly purified β-galactosidase is 38. (b) Cells induced with L-arabinose (a poor inducer) show traces of β-galactosidase and no detectable α-L-arabinosidase (to be expected because of the 30-fold difference in sensitivity). Extracts of β-galactosidase constitutive mutants grown in the presence of succinate and L-arabinose show the same hydrolysis ratio of arabinosides and galactosides as given above. If an active α-L-arabinosidase exists it would have to be induced by galactose but not arabinose, a very unlikely event.

Another possible objection to the induced penetration mechanism hypothesis stems from the fact that α-L-arabinosides are hydrolyzed by extracted enzyme at a smaller rate than β-galactosides. The results with arabinosides could then be interpreted as if the β-galactosidase becomes the rate limiting step at low enzymatic activities. This interpretation is incorrect because whole cells with the same enzyme content, in terms of moles of substrate hydrolyzed per min whether it was a galactoside or an arabinoside, show reduced activity only when β-galactosides were used.

An analysis of the data obtained in the previous experiments from the standpoint of the inducible

penetration mechanism is warranted. Moreover, such analysis yields a forecast of the properties of the hypothetical penetration mechanism which should be useful for its future isolation.

The increase in enzymatic activity, using ONPG, which occurs with organic solvents, lysozyme, polymyxin, heat, or aging of the cell would have to be explained by a nonspecific increase in permeability. This assumption would fit well with the proportional excretion of RNA-breakdown products which occurs concomitantly, indicating a loss of the ability to retain cytoplasm.

Interpreting the results shown in figure 2*B* under the inducible penetration mechanism hypothesis one can conclude: (a) The data conforms with the hypothesis. (b) The β-galactosidase is induced at lower inducer concentration than the penetration mechanism to account for the early part of the curve in which the enzyme content increases more rapidly than the intact cell activity. (c) The penetration mechanism, after a certain period, is induced linearly with inducer concentration, at the same rate as the enzyme. This accounts for the proportional increase of the intact cell activity.

The inhibited enzyme found in extracts cannot be readily explained under any hypothesis because of its presence in extracts of cells which could display activity. The possibility of an artifact is not unlikely.

The experiments with short reaction times performed at Dr. Chance's laboratory do not contradict the inducible penetration mechanism hypothesis. They would indicate that the entry of the substrate by way of the penetration mechanism follows zero order kinetics from its initial velocity.

The inertness of alkali metal ions for the enzyme *in vivo* indicates that, in contrast with the β-galactosidase, the penetration mechanism, which *in vivo* is the rate limiting step, is not influenced by alkali metal ions. Nevertheless, the penetration mechanism needs some salts to function since it is inhibited by triethanolamine buffer.

DISCUSSION

Four hypotheses which could account for the partial expression of the galactosidase *in vivo* have been proposed. Of them, only the one which postulates an inducible penetration mechanism specific for β-galactosides was found compatible with all the data. The experiments which rule out the other three hypotheses are conclusive and the few assumptions used in them were supported by subsequent experimental evidence.

In other instances, specific induction of transport systems, required to bring substrate to the enzyme site, have been postulated. It was used to explain (Barret *et al.*, 1953) the process of citrate utilization in *Pseudomonas fluorescens* and in *Aerobacter aerogenes* (Davis, 1956). Thus, in *A. aerogenes* intracellular accumulation of citrate was shown to occur only after specific induction. Monod and his collaborators have demonstrated directly in *Escherichia coli* a number of specific systems which accumulate amino acids (Cohen and Rickenberg, 1955) and an inducible one which accumulates β-thiogalactosides (Rickenberg *et al.*, 1956). They have postulated an enzymelike "permease" which controls the entrance of the substance. The exit rate of the substance was assumed to depend upon the internal concentration. Thus it is explained how an accumulation occurs and how a steady state between entry and exit is obtained. The "permease" which accumulates β-thiogalactosides has been shown to be induced specifically by the same inducers as the β-galactosidase although it is clearly different from the latter. The different substances accumulated by the system compete with each other in the same fashion as substrates compete for the enzyme.

According to Monod the "permease" mechanism of accumulation can explain the reduced β-galactosidase activity of the whole cell of *E. coli*. The "permease" would be the rate limiting step in the process of bringing the substrate to the β-galactosidase site.

Although Monod's scheme apparently agrees with the results obtained here there are serious discrepancies which must be clarified before drawing conclusions. The accumulation of methyl-β-D-thiogalactoside is inhibited by 2,4-dinitrophenol, azide or by the absence of a source of energy; whereas, the hydrolysis of ONPG is not affected at all under the same conditions. The main evidence obtained by Rickenberg *et al.* (1956) to show the role of the "permease" in the hydrolysis of β-galactosides is the correlation between "permease" and β-galactosidase *in vivo* obtained upon aging of the cells. Both are shown to decrease proportionally. The results obtained here are quite opposite inasmuch as the β-galac-

tosidase activity in the intact cell increases upon aging (table 2).

More recently additional evidence (Cohen and Monod, 1957) for the role of the permease in the hydrolysis of β-galactosides was presented. It is based on the correlation between the two systems in cells with different permease activity as a result of inhibition of the permease synthesis with *p*-fluorophenylalanine.

Whether the discrepancies between Monod's results and ours are real or due to differences in strains or conditions will have to be tested. In view of these differences, the constitutive mutant strain used in our experiments was subsequently examined for the presence of permease and was found to have a constitutive one inasmuch as noninduced cells accumulate radioactive methyl-β-D-thiogalactoside. The strain can be distinguished from the "cryptic" constitutive because it is lactose positive and because of the kinetics of ONPG hydrolysis (Cohen and Monod, 1957). Therefore, the possibility that we have studied a different penetration system than did Monod seems very unlikely.

Assuming that the discrepancies between Monod's results and ours are real, they could be reconciled by postulating two different enzyme-like systems. One would govern the entry of specific substrates into the cell; the second would cause the entered material to accumulate in high concentration. The permeation system would be specifically induced but would not require appreciable energy to function. The substrate brought in by this system could reach the β-galactosidase site or could contact the accumulation system. The accumulation system, though requiring energy, need not be inducible or highly specific in its function to fit our scheme.

A support for this hypothesis comes from recent experiments of Monod (1956). He reported a competition between inducer and inhibitor for a site or substance which remains constant in amount per cell during induction. He concludes that the site or substance must be distinct from either β-galactosidase itself or the permease uptake system. In our scheme this site or substance would correspond to the second enzyme-like system and it would indicate that it is non-inducible.

Off hand, this hypothesis with two enzymelike systems appears very complex. However, if the accumulation system is represented by the cell membrane which could control the exit of the substrate and therefore its accumulation, the hypothesis becomes amenable to test. If the assumption is correct, in the presence of azide or dinitrophenol the rate of exit of an accumulated β-galactoside should increase appreciably. The rate of exit could be determined easily in the absence of external substrate.

To postulate, as Cohen and Monod (1957) have done, that azide or dinitrophenol inhibits "only the energy coupling which allows the permease reaction to function as a pump" serves only to describe the facts.

Thus, the utilization of β-galactosides in *Escherichia coli* would be regulated by the inducible penetration mechanism, a flexible system. It permits the full expression of the enzyme in cells with very little of it or in cells which have entered the stationary phase. Such cells might need the use of greater synthesizing ability to start growing again as is illustrated by the faster rate of RNA, DNA, or protein synthesis in the lag-phase cells. Furthermore, it would indicate that the normal cell synthesizes simultaneously the enzyme and the mechanism which will control its activity.

The smaller quantity of RNA present in stationary cells (Morse and Carter, 1949) can now be explained by the large excretion of RNA in the form of depolymerized products which occurs with age. The remarkable fact that cells can lose most of their RNA and still remain viable poses new questions as to the role of RNA.

For the enzyme chemist it should be of interest that the activating effects of Na and K ions depend upon whether *o*-nitrophenyl-β-D-galactoside or *p*-nitrophenyl-β-D-galactoside is the substrate. With the analogous α-L-arabinosides, the same phenomenon is not observed. For both of these substrates, Na ions activate the enzyme as compared with K ions, although to a different extent in each case.

ACKNOWLEDGMENTS

The author is most grateful to Professors H. A. Lardy and J. Lederberg for their helpful discussions and criticism, and to Professor B. Chance for his valuable cooperation in some of the experiments.

SUMMARY

Four hypotheses were tested to explain the mechanism of the reduced β-galactosidase

activity of whole cells of *Escherichia coli*. Passive diffusion, product inhibition, and inhibitors compounded with the enzyme were ruled out. The data are compatible with the presence of an inducible penetration mechanism. The penetration mechanism is said to be specific for β-galactosides and to be induced together with the β-galactosidase. Its properties differ from those of an inducible system which accumulates β-thiogalactosides in *E. coli*. The penetration mechanism hypothesis offers a flexible regulation system for the enzymatic activity of the cell. The assay of whole cells with α-L-arabinosides permits the bypass of the penetration mechanism.

REFERENCES

BARRET, J. T., LARSON, A. D., AND KALLIO, R. E. 1953 The nature of the adaptive lag of *Pseudomonas fluorescens* toward citrate. J. Bacteriol., **65,** 187–192.

BONNER, D. M. 1955 Aspects of enzyme formation. In *Amino acid metabolism*, pp. 193–197. Edited by W. D. McElroy and H. B. Glass. Johns Hopkins Press, Baltimore.

BOREK, E., RYAN, A., AND ROCKENBACH, J. 1955 Nucleic acid metabolism in relation to the lysogenic phenomenon. J. Bacteriol., **69,** 460–467.

CALIFANO, L. 1952 Liberation d'acide nucléique par les cellules bacteriennes sous l'action de la chaleur. Bull. World Health. Organization, **6,** 19–34.

CHANCE, B. 1956 Interaction of adenosinediphosphate with the respiratory chain. In *Enzymes: units of biological structure and function*, pp. 447–463. Edited by O. H. Gaebler. Academic Press, Inc., New York.

COHEN, G. N. AND MONOD, J. 1957 Bacterial permeases. Bacteriol. Revs., **21,** 169–194.

COHEN, G. N. AND RICKENBERG, H. V. 1955 Existence d'accepteurs specifiques pour les aminoacides chez *Escherichia coli*. Comp. rend., **240,** 2086–2088.

COHN, M. AND MONOD, J. 1951 Purification et propiétés de la β-galactosidase (lactase) d'*Escherichia coli*. Biochim. et Biophys. Acta, **7,** 153–174.

COHN, W. E. 1950 The anion-exchange separation of ribonucleotides. J. Am. Chem. Soc., **72,** 1471–1478.

DAVIS, B. D. 1949 Isolation of biochemically deficient mutants of bacteria by means of penicillin. Proc. Natl. Acad. Sci. U. S., **35,** 1–3.

DAVIS, B. D. 1956 Relations between enzymes and permeability (membrane transport) in bacteria. In *Enzymes: units of biological structure and function*, pp. 509–522. Edited by O. H. Gaebler. Academic Press, Inc., New York.

DEERE, C. J. 1939 On the "activation" of the lactase of *Escherichia coli-mutabile*. J. Bacteriol., **37,** 473–483.

GORNALL, A. G., BARDAWILL, C. J., AND DAVID, M. M. 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., **177,** 751–766.

KAPLAN, J. G. 1954 The alteration of intracellular enzymes. J. Gen. Physiol., **38,** 197–211.

KERR, S. E. AND SERAIDARIAN, K. 1945 The separation of the purine nucleosides from free purines and the determination of the purines and ribose in these fractions. J. Biol. Chem. **159,** 211–225.

KOPPEL, J. L., PORTER, C. J., AND CROCKER, B. F. 1953 The mechanism of the synthesis of enzymes. J. Gen. Physiol., **36,** 703–722.

KUBY, S. A. AND LARDY, H. A. 1953 Purification and kinetics of β-D-galactosidase from *Escherichia coli*, strain K-12. J. Am. Chem. Soc., **75,** 890–896.

LEDERBERG, J. 1950 The β-D-galactosidase of *Escherichia coli*, strain K-12. J. Bacteriol., **60,** 381–382.

LEDERBERG, J. 1951 Genetics studies with bacteria. In *Genetics in the 20th century*, pp. 263–289. Edited by L. C. Dunn. MacMillan Press, New York.

MANSON, L. A. 1953 The metabolism of ribonucleic acid in normal and bacteriophage infected *Escherichia coli*. J. Bacteriol., **66,** 703–711.

MONOD, J. 1956 Remarks on the mechanism of enzyme induction. In *Enzymes: units of biological structure and function*, pp. 7–29. Edited by O. H. Gaebler. Academic Press, Inc., New York.

MONOD, J. AND COHN, M. 1952 La biosyntheses induite des enzymes (adaptation enzymatique). Advances in Enzymol., **13,** 67–119.

MONOD, J., COHEN-BAZIRE, G., AND COHN, M. 1951 Sur la biosynthèse de la β-galactosidase (lactase) chez *Escherichia coli*. La spécificité de l'induction. Biochim. et Biophys. Acta, **7,** 585–599.

MORSE, M. L. AND CARTER, C. E. 1949 The synthesis of nucleic acids in cultures of *Escherichia coli*, strains B and B/r. J. Bacteriol., **58,** 317–326.

NEWTON, B. A. 1956 The properties and mode

of action of the polymyxins. Bacteriol. Revs., **20,** 14–27.

REPASKE, R. 1956 Lysis of gram-negative bacteria by lysozyme. Biochim. et Biophys. Acta, **22,** 189–191.

RICKENBERG, H. V., COHEN, G. N., BUTTIN, G., AND MONOD, J. 1956 La galactosidepermease d'*Escherichia coli*. Ann. inst. Pasteur, **91,** 829–857.

RICKENBERG, H. V., YANOFSKY, C., AND BONNER, D. M. 1953 Enzymatic deadaptation. J. Bacteriol., **66,** 683–687.

ROTMAN, B. 1955*a* A simplified device for continuous growth of microorganisms. J. Bacteriol., **70,** 485–486.

ROTMAN, B. 1955*b* Factors controlling the enzymatic activity of intact cells of *Escherichia coli*. Bacteriol. Proc., **1955,** p. 133.

ROTMAN, B. 1956 On the mechanism of sonic lysis of bacteria. J. Bacteriol., **72,** 827–830.

ROTMAN, B. AND SPIEGELMAN, S. 1954 On the origin of the carbon in the induced synthesis of β-galactosidase in *Escherichia coli*. J. Bacteriol., **68,** 419–429.

SEIDMAN, M. AND LINK, K. P. 1950 *o*-Nitrophenyl-β-D-galactopyranoside and its tetraacetate. J. Am. Chem. Soc., **72,** 4324.

SEVAG, M. G., SMOLENS, J., AND LACKMAN, D. B. 1940 The nucleic acid content and distribution in *Streptococcus pyogenes*. J. Biol. Chem., **134,** 523–529.